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PRINCIPAL INVESTIGATOR: Gerald J. Rowse, Ph.D.

CONTRACTING ORGANIZATION: Mayo Clinic Scottsdale
Scottsdale, Arizona 85259

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Gerald J. Rowse, Ph.D.

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Scottsdale, Arizona 85259

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MUC1 is a large heavily glycosylated membrane mucin glycoprotein which is overexpressed and aberrantly glycosylated by the majority of caricnomas and in particular, by >92% of primary and metastatic breast cancers. It is hypothesized that overexpression of the MUC1 protein by carcinoma cells may confer an advantage upon the cell, perhaps by reducing the adhesive properties of cells and/or by modulating the immunogenicity of the tumor cells. Mice homozygous for the null mutation are healthy, fertile and do not display any obvious pathology. However, Muc-1 deficient mice demonstrate significantly slower growth of polyoma virus middle T antigen induced mammary tumors compared with wild type controls. Depletion of NK cells did not result in significant changes in mammary tumor growth rate in either Muc-1 deficient or wild type mice, but genetic background of the mice complicated evaluation of the study. Overall, these data demonstrate for the first time that Muc-1 expression can significantly affect the growth of breast cancer cells.

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Introduction:

Our goal is to understand the function of the tumor-associated mucin, MUC1, in the progression of cancer in the mammary gland. MUC1 is highly expressed by the majority of cancers and, in particular, by >92% of primary and metastatic breast cancers. The MUC1 protein is a large, rod-like molecule that projects far from the cell surface as a long filament. The protein core is extensively glycosylated through O-glycosidic linkage to serine and threonine, with as much as 50 to 90% of its molecular mass made up of oligosaccharide side chains. This contributes significantly to the rigidity of the molecule. MUC1 is expressed on normal epithelial tissues at low levels. Appearance of MUC1 correlates closely with epithelial differentiation in various organs and is detected well before the organs are functional. The presence of the large, highly extended molecule of MUC1 on the surface of epithelia suggests that it may act as a physical barrier protecting the cells. MUC1 may be involved in epithelial morphogenesis, perhaps acting to mask adhesive molecules present on the cell surface and aiding in the formation of a lumen. When epithelial tissues become cancerous, MUC1 expression is increased at least ten fold, and the glycosylation and spatial distribution of the protein at the cell surface are altered. MUC1 in normal polarized epithelia is expressed only at the apical side of lumens and ducts. However, in many adenocarcinomas polarization is lost, and the protein is found over the entire surface of the cells. Our hypothesis is that expression of this protein benefits tumor cells and their metastatic counterparts, perhaps by altering the adhesive properties of cells or by providing a protective layer around cells that may shield them from immune surveillance.

The ability to create mice that possess deficiencies in specific genes is providing important insights into the physiological role played by specific proteins during embryonic and postnatal development and during adult life. The expression pattern of the *Muc-1* gene in the adult and embryo of the mouse is similar to that of the human (the human gene designation is *MUC1*; the mouse gene is *Muc-1*) (Braga et al., 1992). Muc-1 expression is also elevated in mouse mammary gland tumors. Since mammary gland cancer in the mouse closely resembles human breast cancer and expression patterns are similar, our experiments should enable us to analyze the functional role of Muc-1 in the development and progression of cancer. To investigate the biological function of the Muc-1 protein we disrupted the *Muc-1* gene using homologous recombination in mouse embryonic stem cells. Mice were generated that lacked expression of the Muc-1 protein. We and others had postulated that Muc-1 on the apical surface of differentiating epithelial cells may repel adjacent cells or mask adhesive molecules, thus promoting the formation of a lumen. However, we were surprised to find that, despite the

widespread expression of Muc-1 during epithelial organogenesis, mice lacking Muc-1 protein were born at the expected frequency and appeared normal in all respects.

Direct evidence of a role for MUC1 in the development and progression of breast cancer has not been demonstrated previously. In many cancer cells polarization of the epithelial cells is lost and the MUC1 protein can be detected on all cell surfaces, including those facing the stroma and adjacent cells. Under these circumstances, the anti-adhesive property of MUC1 may destabilize cell-cell and cell-substratum interactions, thus promoting the disaggregation of a tumor site, leading to tumor spread and metastasis. Previous studies have suggested various possible roles for the MUC1 mucin in facilitating tumor growth, including inhibition of cell-cell contacts, protection from recognition and destruction by immune cells, and also serving as an E-selectin ligand to facilitate escape of metastatic cells from the blood stream. Thus overexpression of the Muc-1 molecule could provide many potential benefits to tumor cells.

We are currently using Muc-1 deficient and control mice to investigate the role of the Muc-1 molecule in normal development and in the development and progression of breast cancer in mice. The following progress was achieved in two years of the study:

Mice homozygous for the *Muc-1* mutation have been bred onto inbred 129SV and C57Bl/6J (N8) lines and have been demonstrated to be healthy, fertile and viable. In all cases, animals homozygous for the disrupted *Muc-1* allele (-/-) were obtained at the expected Mendelian frequency of 1:2:1 (Wild type : Heterozygotes : Homozygous Mutants). Loss of Muc-1 expression did not appear to effect organogenesis as examination of hematoxylin-eosin stained sections prepared from all the major organs revealed no obvious differences between Muc-1 deficient mice and their corresponding litter mates. Muc-1 deficient mice failed to demonstrate significant upregulation of expression of mucin-like genes or membrane glycoproteins including Muc-2, Muc-4, ASGP-2, CD34, CD43 (leukosialin), glycophorin and MadCAM-1.

We have demonstrated for the first time that the Muc-1 molecule facilitates the growth of breast tumors in mice transgenic for the polyoma virus middle T antigen (MTag). Although similar numbers of mice developed tumors by 4 months of age in both Muc-1 deficient and control groups, tumor growth rate was significantly decreased in mice homozygous for the *Muc-1* mutation when compared with wild type control mice (Figure 1). In addition, there was a trend towards decreased lung metastasis in Muc-1 deficient mice. Overall, 58% of mice developed grossly observable lung metastases, with 53% of *Muc-1* -/- mice and 67% of *Muc-1* +/+ mice developing metastases. Based on the sample sizes in this study, the power to detect a statistically

significant difference in the rate of metastasis between the two groups was only 33%. It is possible that with a larger sample size, this difference in metastatic rate would be statistically significant.

The presence or absence of Muc-1 in tumors did not significantly affect tumor growth rate or apoptosis. Tumor growth rate was estimated by two independent methods and found to be equivalent between Muc-1 deficient and wild type animals. *In vivo* studies measured the incorporation of BrdU into mammary tumors growing in Muc-1 deficient or wild type mice. *In vitro* studies measured the proliferation of cells isolated from middle T antigen induced mammary tumors that developed in Muc-1 deficient and wild type mice. Similarly, rates of apoptosis in mammary tumors, as demonstrated by the TUNEL assay, did not differ in mammary tumors induced in Muc-1 deficient and wild type mice transgenic for MTag. One possible explanation for these findings is that the assays are not sensitive enough to detect subtle differences in proliferation rates or apoptosis. This finding seems unlikely as it has recently been demonstrated that rates of apoptosis and cell proliferation differ significantly between *ras*- and *myc*-induced mammary tumors in MMTV-*ras* and MMTV-*myc* transgenic mice (Hundley, et al., 1997). However, it remains difficult to prove that subtle changes in the rates of cellular proliferation and/or apoptosis are not occurring in this system.

A study was initiated to investigate the effect of *Muc-1* gene mutation on tumor development and progression induced by the *neu* protooncogene. To ensure that the outbred genetic background of animals required for the study would not affect the growth of the *neu* proto-oncogene induced mammary tumors, *neu* transgenic mice (FVB strain) were backcrossed on to B6 mice and tumor growth of the offspring were compared to that seen in inbred FVB mice. Unfortunately, tumor growth was significantly altered in FVB/B6 F1 mice. Inbred virgin FVB female mice begin to develop tumors at about 7 months of age and have a 65% tumor incidence by 12 months of age. In contrast, tumors were not observed in F1 mice until 18 months of age. The F1 mice had a 5% tumor incidence at 18 months of age (2/50). This delay in tumor incidence suggests that a dominant acting gene(s) present in the B6 genome can modulate the growth or induction of *neu* induced mammary tumors. Clearly, the effect of the B6 genome on *neu* proto-oncogene induced mammary tumor growth would mask any effect of Muc-1 overexpression on tumor growth. However, as overexpression of the *neu* proto-oncogene is observed in 30% of human breast cancers, other genetic loci which could effect tumorigenesis by the *neu* gene are clearly of interest.

Overall studies to date have substantiated the hypothesis that overexpression of Muc-1 in tumor cells facilitates the growth of breast cancer. However, it is not currently clear how the overexpression of Muc-1 facilitates tumor growth. Lack of Muc-1 expression did not noticeably affect the rate of cellular proliferation or apoptosis in mammary tumors induced by MTag. Similarly, a role for Muc-1 in the development or functioning of normal epithelial glands has not been demonstrated by the Muc-1 deficient mice. These animals appear normal, healthy and fertile. We have continued to investigate the role of Muc-1 in organogenesis, mammary tumor development and metastasis, in addition we are investigating the role of genetic influences on tumorigenesis induced by the overexpression of the *neu* protooncogene.

Methods and Materials:

Mammary Gland Whole Mount Analysis: Muc-1 deficient and wild type mice inbred on a C57Bl/6 background were terminated at 4, 5 and 6 weeks of age. All animals were housed in close proximity to allow synchrony of estrous cycles. Auxiliary and inguinal mammary glands were removed, spread on glass slides and fixed in acetone overnight. The tissues were subsequently cleared by passage through xylene and a graded series of alcohols (100, 95, 80, 70 and 40%). The tissues were subsequently stained for 20 min in Mayer's hematoxylin and destained in ammonium water (3.4mM NH_4OH). To analysis the extent of mammary gland development, tissues were photographed under a dissecting microscope, the images scanned into the computer and the percentage of the total mammary fat pad occupied by ductal elements was measured.

In Vivo Depletion of Natural Killer Cells: For the study, outbred (C57Bl/6 x 129sv) Muc-1 deficient and wild type male mice containing the MTag transgene were crossed to Muc-1 deficient or wild type C57Bl/6 females to ensure that all experimental mice were at least 50% C57Bl/6. It was important that all mice be at least 50% C57Bl/6 as the epitope recognized by the NK1.1 antibody is only expressed on C57Bl/6 mice. Mice were weaned at 3 weeks of age and randomly assigned to either NK1.1 or vehicle-injected groups (n = 20 per group) and housed 4 - 5 mice per cage. Mice were injected with 100 ul of antibody or PBS at weekly intervals beginning at 4 weeks of age.

Natural Killer Cell Assay: Eighteen hours before the assay mice were injected i.p. with 100ug of polyinosinic:polycytidylic acid (Sigma, St. Louis, MO) to stimulate NK cell activity. For the assay, mice were terminated, spleens aseptically removed and dissociated in RPMI medium (Grand Island Biochemical Co., Grand Island, NY). Red blood cells were lysed using Tris-NH_4Cl. Viable cells were counted and resuspended in RPMI supplemented with 10% fetal

bovine serum (Gibco) and 10 mM HEPES (Sigma) at a concentration of 1.5 x 10^7 cells/ml of medium. Yac-1 lymphoma cells were labeled with 500 uCi sodium [^{51}Cr] chromate/10^7 cells (Amersham, Arlington Heights, IL) for 90 min, washed and resuspended at 10^5 cells/ml of medium. Cell suspensions of 100 ul were added to 96 well V-bottomed plates (Rainin Instrument Co., Woburn, MA). Spleen cells were added to each well to produce effector to target cell rations of 150:1, 75:1, 37.5:1, 17:1, 9:1, 4.5:1. Plates were incubated for 6h at 37°C in 95% air, 5% CO_2 and [^{51}Cr] release was from lysed target cells was determined by gamma counting. The percent of specific [^{51}Cr] released at each effector to target cell ratio was computed using the formula:

$$\% \text{ Specific Lysis} = \frac{\text{Test cpm - Spontaneous cpm}}{\text{Total cpm - Spontaneous cpm}} \times 100.$$

Where Test cpm = counts in experimental cultures of target cells and effector cells.

Spontaneous cpm = counts in cultures containing only target cells.

Total cpm = counts obtained by adding 100 ul of 1N HCl to target cells to lyse all cells.

Tumor Measurement: For all studies, female mice were weaned at 3 weeks of age and housed in groups of 4 to5 animals per cage. Mice were palpated on a biweekly basis for the appearance of tumors. Once tumors appeared tumor growth was measured with calipers and tumor weight was calculated as follows: $g = \frac{(l \times w^2)}{2}$.

Immunohistochemistry: Tumors and normal mammary glands were obtained from pure strain FVB and FVB/B6 F1 *neu* transgenic mice. Tissues were fixed in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid), paraffin embedded, and sectioned for immunohistochemical analysis. Briefly, endogenous peroxidase activity was decreased by incubating the tissue sections with 0.6% H_2O_2 in PBS (pH 7.4) for 30 minutes at 25°C. After washing with PBS pH 7.4, sections were incubated in 1X antigen retrieval buffer at 95°C for 5 minutes. Sections were then incubated in 0.05% saponin (Sigma) in distilled water for 30 min at 25°C . After washing with PBS pH 7.4 3 times, sections were blocked with 50% fetal calf serum and stained with Ab-3 murine monoclonal antibody that recognizes the *neu* oncogene (Oncogene Science,Uniondale, NY; diluted 1:100), for 1 hr at 25°C. Following three 5 minute washes with PBS pH 7.4, the sections were incubated with peroxidase-conjugated rabbit anti-mouse antibody (Dako Corporation, Carpinteria, CA, diluted 1:50) for 1 hr at 25°C. Following three 5 min

washes, the substrate solution consisting of 0.03% hydrogen peroxide in PBS and 1 mg/ml diaminobenzoate (Sigma) was added, and the color allowed to develop for 5-8 min. Following color development, the reaction was quenched by washing the slide in distilled water. The tissue sections were counterstained lightly with hematoxylin.

Western Blotting: Mammary tumor tissues were rapidly frozen in liquid nitrogen and stored at -80°C until use. Tissues were lysed and sonicated (Branson Microtip,) in a solution comprising 0.05M sodium chloride, 0.02M Tris HCl pH 7.4, 100ug/ml leupeptin, 50ug/ml aprotinin and 1% NP-40 (all reagents were from Sigma). Equivalent amounts of protein were separated onto 5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond C++,). Membranes were stained with ponseau S and photographed. Nitrocellulose membranes were blocked in 5% non-fat dry milk in PBS + 0.05% Tween 20 and probed with either anti-neu antibody (Ab-3, Oncogene Science, diluted 1 in 2500) or anti-actin antibody (CP-01, Oncogene Research, Cambridge, MA, diluted 1 in 1000) for 1 hr at 25°C. Membranes were incubated with 2° antibody (HRP conjugated rabbit anti-mouse antibody, Dako, diluted 1 in 2000) for 1 hr at 25° C. The membranes were visualized by ECL (Amersham).

Statistics: Latencies of *neu*-induced mammary tumor development were plotted using Kaplan-Meier survival plots and compared by Chi-squared analysis. The number of unlinked genetic controlling *neu*-induced tumor latencies of mammary tumors induced by the over-expression of the *neu* proto-oncogene was estimated using the method of Dietrich et. al. (Dietrich et al., 1993; Wright, 1968). This method assumes that the average of the mean latency for the F1 and the FVB mice equals the mean latency for the F1B1 mice and that the distributions be normally distributed with equal variances. Our data for tumor latency meet these criteria.

Results:

Specific Aim 1: Analysis of the effects of Muc-1 gene mutations on epithelial organogenesis.

Our previous studies failed to indicate a loss of viability or defects in the organs of mature Muc-1 deficient mice. However, it is possible that subtle defects in the organogenesis may still occur. To investigate this possibility, mammary gland organogenesis in Muc-1 deficient and control animals was studied. The mammary gland is an organ system which develops late, maturing in the juvenile mouse and it is not required for survival of the individual. Thus it is an excellent system in which to investigate possible subtle changes in organogenesis in Muc-1 deficient mice. Preliminary experiments suggested that ductal elements developed more rapidly in Muc-1 deficient mice than in wild type control mice, but that similar levels of ductal

development were achieved in adult mice of both groups. However, the small sample sizes (n=3 per group per time point) in this trial may not be representative of the total population. Thus a larger study was undertaken to investigate the role Muc-1 in the development of the mammary gland. For the study, virgin female C57Bl/6 mice of the Muc-1 deficient and wild type strains were terminated at 4, 5 and 6 wk of age. Inguinal and auxiliary mammary glands were removed, prepared for whole mount staining and the extent of ductal invasion of the mammary pad was evaluated. At four weeks of age, ductal elements occupied a greater percentage of the mammary fat pad in Muc-1 deficient mice compared with wild type mice (Figure 2, $p<0.01$). By five and six weeks of age there were no significant differences in the extent of invasion of the mammary fat pads by epithelial elements (Figure 2).

Specific Aim 2: Analysis of the effects of Muc-1 gene mutation on tumor formation and progression.

We have demonstrated that overexpression of Muc-1 facilitates tumor growth of MTag-induced mammary tumors. However, the mechanism by which Muc-1 overexpression facilitates tumor growth is not clear. It appears likely that expression of Muc-1 does not strongly affect the rate of cellular proliferation or apoptosis in this tumor model. It is possible that the overexpression of Muc-1 facilitates mammary tumor growth by blocking the ability of immune effector cells to recognize and lyse tumor cells. This hypothesis is supported by the findings of Wiel-van Kemenade et al. (1993), who demonstrated that tumor cells transfected to overexpress MUC1 were resistant to lysis by both Natural Killer (NK) cells and cytotoxic T lymphocytes compared to non-transfected parental cells. A study was undertaken to investigate if increased lysis of tumor cells by the cells of the immune system was responsible for the decreased tumor growth rate observed in Muc-1 deficient mice.

Initial studies have focused on the role of natural killer cells in this tumor model. Natural killer cells are large granular lymphocytes with the ability to spontaneously recognize and lyse a variety of malignant and transformed cells. They are thought by many to be the bodies first line of defense against tumor cells. To investigate if increased NK cell recognition and lysis of tumor cells in Muc-1 deficient mice is responsible for the slower tumor growth rates observed in Muc-1 null mice in our model, NK cell activity was depleted by repeated injections of the NK1.1 antibody (Seaman et al., 1987). A single injection of NK1.1 antibody resulted in complete loss of NK cell activity for at least 7 days (Figure 3a). Repeated injections of NK1.1 antibody at one week intervals resulted in the sustained abrogation of NK cell activity, as indicated by the lack of NK cell activity in mice after 3 months of injections (Figure 3b). Tumor growth in animals treated with NK1.1 antibody did not differ significantly compared with animals injected with

vehicle alone in either Muc-1 deficient or wild type mice (Figure 4, $p>0.2$). Further, there was also no difference in tumor growth rates between Muc-1 deficient and wild type mice in the vehicle-injected conditions. Thus the vehicle-injected groups failed to replicate the finding that tumors grow significantly more slowly in Muc-1 deficient mice than in wild type mice. Further, variability within a group was significantly greater than variability between groups. These findings suggested that MTag-induced tumor growth rate could be affected by the genetic background of the animal in a fashion similar to that observed in *neu* transgenic mice (July 1996 progress report). Genetic influences appeared to have a greater affect on tumor growth rate than did treatment with NK1.1 antibodies, making it difficult to analyze the role of NK cells in mediating the decrease in tumor growth rate observed in Muc-1 deficient mice.

To investigate the effect of genetic background on MTag-induced tumor growth rate, pure strain FVB males containing the MTag transgene were crossed onto FVB, C57Bl/6 or 129sv females. Tumor growth rates were followed for 80 days in the resulting MTag transgene positive female offspring. MTag-induced tumor growth rates were not significantly different in pure strain FVB mice($n=22$) and FVB x 129sv F1 mice ($n=31$) (Figure 5), while tumors in FVB x C57Bl/6 F1 mice ($n=11$) grew significantly slower than those in either FVB or FVB x 129sv F1 mice (Figure 5, $P<0.01$). Thus C57Bl/6 mice contain genetic elements that significantly suppress tumor growth induced by the expression of the MTag transgene. The effect of C57Bl/6 genome on MTag-induced tumor growth could have serious implications for studies of the role of Muc-1 overexpression in MTag-induced mammary tumors. The initial study of Muc-1s role in MTag-induced mammary tumors used outbred (C57Bl/6 x 129sv) mice and, although every attempt was made to ensure that Muc-1 deficient and wild type control mice had equivalent genetic backgrounds, the results of the study could have been influenced by variations in genetic background of the mice. To examine this issue, all animals in the study that exhibited rapid tumor growth rate patterns indicative of the pure strain FVB (or 129sv) genetic background were removed and the data was reanalyzed. Animals were removed from the data subset if their tumor displayed signs of rapid early growth; tumor burden exceeded 0.3 grams at 90 days of age (11 Muc-1 deficient mice and 8 wild type mice). On reanalysis of the data, tumor growth was still significantly slower in Muc-1 deficient mice than in wild type control mice at 125 days of age (Figure 6, $p<0.01$). Thus we conclude that the initial observation that the overexpression of Muc-1 significantly facilitates tumor growth is valid.

Specific Aim 3: Analysis of the effect of Muc-1 gene deletion on metastasis of mammary gland tumors.

In previous a study, the overexpression of Muc-1 on MTag-induced mammary tumors resulted in a trend towards increased lung metastasis compared with metastasis in Muc-1 deficient mice (67% vs 53%, $p>0.12$). With 35 animals in the control group and a 14% difference in metastasis between the two groups, the power to statistically detect such a difference is only 33%. Thus, the data suggest that Muc-1 overexpression could facilitate tumor metastasis, but further research is required to substantiate this difference. As was mentioned previously, one potential role of Muc-1 overexpression in tumors is to shield the tumors from recognition by the immune system. It has been demonstrated that NK cells play an important role in controlling metastatic spread and it has been shown that overexpression of Muc-1 in vitro decreases the ability of NK cells to recognize and lyse tumor cells. Thus it is possible that one role of Muc-1 overexpression is to protect tumors cells from lysis by NK cells during the metastatic cascade. To test this hypothesis, rates of metastasis were compared between Muc-1 deficient and wild type control mice treated with either anti-NK antibodies (NK1.1) or vehicle (n=20 mice per group). Depletion of NK cell activity did not result in a change in the rate of metastasis in either Muc-1 deficient or wild type mice (Figure 7). This finding could suggest that NK cells do not play a significant role in limiting the metastasis of MTag induced mammary tumors. However, as discussed above, the animals in this study are of an outbred genetic background and it is possible that the outbred background affected the rate of lung metastasis independent of NK cell activity.

Specific Aim 4:

We have demonstrated that genetic background significantly affects mammary tumor induction in transgenic mice that overexpress the unactivated *neu* protooncogene in the mammary gland under the control of the MMTV 3'LTR promoter (July 1996 Progress Report). Tumor latency was significantly increased in FVB x C57Bl/6 F1 (F1) mice compared with pure strain FVB mice (Figure 8, $p<0.001$). Tumor latency in [FVB x C57Bl/6] F1 x FVB backcross mice (F1B1) was intermediate between that observed in pure strain FVB and F1 mice and was significantly different from both groups (Figure 8, $p<0.001$). Analysis of the distribution of tumor latencies in the 3 groups of animals (Figure 9) allowed an estimate of the number of genetic loci involved in modulating *neu*-induced mammary tumorigenesis. Development of mammary tumors in FVB and F1 mice was only affected by environmental variance as the animals are genetically identical, while tumor latencies in the F1B1 mice were affected by both genetic and environmental variance as allelic differences are segregating in these mice. The data suggest that genetic variance accounts for approximately 88% of the total variance in tumor

latency in the F1B1 mice. Analysis of the genetic variability in latency for *neu*-induced mammary tumors suggests that 2.7 independent loci may be involved in modulating *neu* induced tumorigenesis.

To investigate the possibility that the altered tumor latencies observed in this study could result from differences in the level of neu expression, neu expression levels were compared in the mammary glands of virgin female FVB and F1 *neu* transgenic mice at three months of age. Neu protein was expressed in the epithelial elements of mammary glands in both FVB and F1 mice (Figure 10). The expression of the neu proto-oncogene was variable, with some cells expressing strongly, while others expressed the protein weakly or not at all. Interestingly, variations in the levels of neu expression were greater in cells from a single mouse than they were between mice of the FVB and F1 strains.

Although tumor development was significantly delayed in F1 mice, it was of interest to investigate if the C57Bl/6 genotype also affected tumor growth rate. Interestingly, when tumor growth rates were compared, ignoring the effect of tumor latency, tumors developing in F1 mice grew faster than tumors developing in pure strain FVB mice. To directly compare tumor growth rate in these two groups, the data was logarithmically transformed and a best fit linear regression was estimated for each tumor. When the slopes of these lines were compared, tumors in F1 mice grew significantly faster than tumors growing in pure strain FVB mice (Figure 11, $p<0.01$).

To investigate the possibility that the increased tumor growth rates observed in F1 mice were due to altered levels of expression of the *neu* oncogene, tumors from both groups were stained for the presence of neu. Tumors derived from both FVB and F1 mice stained intensely for the neu oncogene. To further quantitate the level of neu expression in these tumors, western blots were performed using anti-neu antibodies. Once again there were no significant differences in the levels of neu expression between tumors derived from FVB and F1 mice (Figure 12).

Conclusions:

We have demonstrated that mice homozygous for the Muc-1 mutation are healthy, fertile and do not exhibit any deleterious effects in two different strains of mice. The present studies suggest that *Muc-1* mutation may alter the organogenesis of the mammary gland, as there was greater penetration of ductal elements into the mammary fat pads in Muc-1 deficient mice at four weeks of age. Currently the importance of the increased mammary gland development in Muc-1

deficient mice is unclear. The difference in mammary gland development between wild type and Muc-1 deficient mice is no longer apparent by 5 weeks of age, suggesting that it is of little functional consequence for the mice. It may reflect an earlier start or increased rate of organogenesis in the mammary glands of Muc-1 mutant mice. Examination of the extent of ductal element penetration of mammary fat pads in 3 week old females will be performed.

These studies demonstrate for the first time that the Muc-1 molecule facilitates growth of breast tumors in mice transgenic for the MTag. Tumor growth rate was significantly decreased in mice homozygous for the Muc-1 mutation when compared with wild type control mice. Interestingly, the tumors did not exhibit different rates of proliferation or necrosis as measured by BrdU incorporation or in vitro proliferation. These findings suggest that facilitation of tumor growth induced by the overexpression of the Muc-1 molecule by mammary tumor cells does not involve large changes in rates of cellular proliferation or apoptosis. However, it is possible that small changes in the rates of cellular proliferation and/or apoptosis which are below the sensitivity of the current assay system to detect could result from the overexpression of Muc-1 by mammary tumor cells. Such subtle changes in cell growth or death rates could be sufficient to account for the observed differences in tumor growth observed in this model given the exponential nature of tumor growth.

Another potential mechanism by which Muc-1 overexpression could affect tumor development and metastasis is by modulating the immunogenicity of mammary tumor cells. It has been demonstrated that the overexpression of MUC1 *in vitro* by transfecting tumor cells to express high levels of MUC1 protein results in protection from lysis by natural killer cells and cytotoxic T lymphocytes (Wiel-van Kemenade et. al., 1993). It has been speculated that the presence of the large negatively charged MUC1 molecule on the tumor cell surface blocks access of immune cells to tumor specific antigens and other cell adhesion molecules present on the tumor cell surface. Thus it is possible that overexpression of Muc-1 by mammary tumor cells facilitates tumor growth by blocking tumor recognition and lysis by natural killer cells and cytotoxic T lymphocytes. To investigate the potential role of Muc-1 overexpression in protecting tumor cells from destruction by the immune system, we have studied the role of NK cells. We have demonstrated that injection of the monoclonal antibody NK1.1 on a weekly basis can maintain suppression of NK cell activity for a three month period. However, studies into the role of NK cells in decreasing tumor growth rate in Muc-1 deficient mice were hampered by the effects of genetic background on MTag-induced mammary tumors. The study must be repeated using mice with a defined (FVB x C57Bl/6) F1 background. This study is currently underway.

We have demonstrated that induction of mammary tumors by overexpression of the *neu* proto-oncogene was significantly affected by genetic elements of C57Bl/6 mice in a dominantly acting fashion. This suggests that C57Bl/6 mice may contain tumor suppresser genes that affect mammary tumorigenesis induced by the *neu* proto-oncogene. We have recently reported that *neu* induced mammary tumors developing in FVB x C57Bl/6 mice undergo loss of heterozygousity at chromosomes 3 and 4 with high frequency (Ritland et al., 1997). Further, there is preference for the loss of the B6 allele in 78% of the LOH cases studied. Together data these suggest that the C57Bl/6 mice contain one or several tumor suppresser genes that regulate mammary tumor induction by over-expression of the *neu* proto-onocogene.

Alternately, it is possible that the MMTV 3' LTR promoter used to drive mammary specific over-expression of the *neu* proto-oncogene in these transgenic mice was less efficient at inducing gene expression in C57Bl/6 mice compared with FVB mice. If the MMTV 3' LTR were less active in C57Bl/6 mice, then F1 mice would express lower levels of the neu proto-oncogene in their mammary glands and could be at lower risk for developing mammary tumors. Recently it has been reported that ras expression in MMTV-ras transgenic mice correlates with MMTV 3' LTR promotor methylation status and that the long tumor latencies observed in these mice are associated with an age dependent demethylation of this promoter (Mangues et al., 1995). Further, studies have demonstrated that mice have strain specific modifiers of methylation and that C57Bl/6 mice are more efficient at methylating transgenic DNA than are SJL or DBA/2 strains of mice (Engler et al., 1991). However, studies in transgenic mice utilizing the MMTV 3' LTR have not previously addressed the issue of level of transgene expression in different strains of mice. The present study demonstrates that levels of neu protein appear to be similar in mammary glands of virgin female FVB and F1 mice, suggesting that modulation of MMTV promoter activity may not be responsible for the increased tumor latencies observed in this study. This conclusion is further supported by the finding that mammary tumors induced in transgenic mice by the MMTV-myc and MMTV-ras transgenes are not affected by the presence of the C57Bl/6 genome. In this study, the authors report that tumor latency in these transgenic mice did not significantly differ if the transgene was expressed on a pure strain FVB or an FVB x C57Bl/6 x Balb/c outbred background. Thus, while we cannot conclusively rule out a role for strain specific differences in MMTV promotor methylation, it would appear that mammary tumor induction by the over-expression of the *neu* proto-oncogene is influenced by the presence of tumor suppresser genes present in C57Bl/6 mice.

The finding that mammary tumor growth rates are increased in F1 mice compared with pure strain FVB mice is of interest. As tumors from F1 and FVB mice express similar levels of

the neu protein, changes in the level of *neu* expression do not appear to underlie this phenomena. One possible explanation of this finding is that F1 mice are considerably older that pure strain FVB mice when their mammary tumors arise and age related loss of suppressive influences from the surrounding normal tissues could occur in F1 mice. Alternately, it is possible that genetic changes required to induce mammary tumors in F1 mice result in the development of a more aggressive tumor phenotype. In support of this hypothesis, we have demonstrated that F1 mice preferentially lose the B6 allele of chromosomes 3 and 4 (Ritland et al.,1997). As chromosome 4 has been reported to contain a number of tumor suppresser genes, it is possible that loss of these genes results in a more aggressive tumor.

Future Directions:

Mixed genetic backgrounds have complicated the interpretation of several studies in this project. To complete the projects proposed in the revised statement of work, I have requested an unfunded extension of the project. I will repeat the NK depletion studies using mice of a uniform genetic background. I am currently backcrossing Muc-1 knockout mice onto the FVB background. Although I cannot backcross the mice the 20 generations required to achieve true inbred status, I am taking several steps to minimize the effect of any residual outbred genetics. First, I am using the Muc-1 knockout mice on an inbred 129sv background as a starting point for the backcrosses as our studies indicate that there is little to no difference in MTag induced tumor latencies between FVB and 129sv mice. Second, backcrosses are through a single male at each generation to minimize the variability in129sv genes that are passed to successive generations. Using this strategy, I am currently on backcross #4 (93.75% FVB). I estimate that by October 1997 I will have mice at 98.44% FVB. These mice will only have ~1.6% 129sv alleles and the contribution of 129sv alleles will be uniform in the mice. Furthermore, the effect of the 129sv genome on MTag induce mammary tumorigenesis is minimal at best. Thus, by October of 1997, I will be ready to begin the studies on the role of NK cells in decreasing tumor growth rate in Muc-1 deficient mice.

The studies described in this report utilize the unique strength of the *Muc-1* mutant mouse model to investigate the role of the Muc-1 molecule in organogenesis, tumor development and progression and in tumor metastasis. These are the first studies to directly demonstrate a role for Muc-1 overexpression in facilitating the growth of breast cancer *in vivo*. It is hoped that in the long term the data derived from these studies could be used to improve the treatment of human breast cancer. Data also demonstrated C57Bl/6 mice contain genetic elements that suppress the

ability of *neu* proto-oncogene overexpression to induce mammary tumors in *neu* transgenic mice. It is likely that FVB mice contain mutant or defective copies of these genes, thus allowing *neu*-induced tumor development to occur at a more rapid rate. The identification of these tumor suppresser genes could be of great importance as the *neu* gene is over-expressed in approximately 25% of human breast cancers.

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Figure Legends:

Figure 1. Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth in Muc-1 Mutant and Wild Type Mice.

Rate of polyoma virus middle T antigen-induced mammary tumor growth in Muc-1 deficient (filled squares, n=81) and wild type (open circles, n=35) mice. At 104 days of age, Muc-1 deficient mice had significantly smaller tumors than did wild type mice ($p<0.05$). By the 124 day end point, differences in tumor size were highly significant ($p<0.001$). Asterisks indicate statistical significance.

Figure 2. Mammary Gland Organogenesis in Muc-1 Deficient and Wild Type Mice.

Muc-1 deficient and wild type virgin female mice were terminated at 4, 5 and 6 weeks of age. Mammary glands were removed, spread on glass slides, fixed in acetone overnight and stained with Mayer's hematoxylin. Equivalent auxiliary mammary glands were viewed and photographed under identical conditions. The area of the mammary fat pad occupied by ductal elements was calculated and averaged for each group of mice. Bars represent mean ± standard error margin.

Figure 3. Effect of Injection of NK1.1 Antibody on Natural Killer Cell Activity.

In vitro measurement of NK cell activity following ip injection of 75ul of monoclonal antibody NK1.1 ascites. (A) Mice were injected with either PBS or NK1.1 antibody and terminated at the time points indicated (3 mice/group/timepoint) and the ability of splenic lymphocytes to lyse [^{51}Cr] labelled Yac1 target cells in a 6 hr cytotoxicity assay at an effector to target cell ratio of 150:1. A single injection of NK1.1 antibody (filled square) resulted in a sustained suppression of NK cell activity for 7 days relative to PBS injected control mice (circles). (B) The ability of splenic lymphocytes to lyse [^{51}Cr] labelled Yac1 target cells in a 6 hr cytotoxicity assay at an effector to target cell ratio of 150:1 was measured in mice injected with PBS (n=2), injected one time with NK1.1 antibody (n=1) or injected weekly with NK1.1 antibody for 3 months (n=3). Mice were injected 3 days prior to termination and measurement of NK cell activity. This assay demonstrates the ability of repeated injections of NK1.1 to maintain the suppression of NK cell activity for up to 3 months.

Figure 4. Role of Natural Killer Cells in Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth.

Rate of polyoma virus middle T antigen-induced mammary tumor growth in Muc-1 deficient (open symbols) and wild type (filled symbols) mice. Virgin female mice were weaned at 3

weeks of age and housed in groups of 4 to 5 mice per cage. Beginning at 4 weeks of age, mice were injected with either 75ul of NK1.1 ascites (circles) or with PBS (squares). Tumor growth was followed on a weekly basis until the mice were 121 days old. Tumor growth was not significantly different between NK1.1 and PBS-injected mice in either Muc-1 deficient or wild type mice.

Figure 5. The Effect of Genetic Background on Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth.

Mammary tumor growth was followed for 80 days in polyoma virus middle T antigen transgenic mice of pure strain FVB (n=22, square), FVB x 129sv F1 (n=31, circle) or FVB x C57Bl/6 F1 (n=11, triangle). Tumor growth rate was not significantly different between FVB and FVB x 129sv F1 mice, while tumors in FVB x C57Bl/6 mice grew significantly more slowly than did tumors in FVB mice ($p<0.01$).

Figure 6. Reanalysis of Polyoma Virus Middle T Antigen-Induced Mammary Tumor Growth in Muc-1 Mutant and Wild Type Mice.

To correct for possible influences of variations in genetic backgrounds of mice in the original study (figure 1), mice that exhibited rapid tumor growth rate patterns indicative of the pure strain FVB (or 129sv) genetic background were removed from the data set and the data were reanalyzed. Animals were removed from the data set if their tumors displayed signs of rapid early growth; tumor burden exceeded 0.3 grams at 90 days of age (11 Muc-1 deficient mice and 8 wild type mice). On reanalysis of the data, tumor growth was still significantly slower in Muc-1 deficient mice (filled square, n=70) than in wild type control mice (open circle, n=27) at 124 days of age (Figure 6, $p<0.01$).

Figure 7. Lung Metastasis in Muc-1 Deficient and Wild Type Mice Injected with NK1.1 Antibody or Vehicle.

Rates of metastasis were compared between Muc-1 deficient and wild type control mice treated with either anti-NK antibodies (NK1.1) or vehicle (n=20 mice per group). Mice were injected with NK1.1 or PBS at weekly intervals beginning at 4 weeks of age. Mice were terminated at 121 days of age and their lungs removed, fixed in methacarn and examined under a dissecting microscope for lung metastasis. Depletion of NK cell activity did not result in a change in the rate of metastasis in either Muc-1 deficient or wild type mice

Figure 8. Tumor Incidence in *Neu* Proto-Oncogene Transgenic Female Mice.
Inbred FVB virgin female mice transgenic for the *neu* proto-oncogene developed mammary tumors between 7 and 12 months of age with a 70% incidence at 12 months of age. In contrast, at 18 months of age only 5% of FVB x C57Bl/6 F1 transgenic females developed mammary tumors. When F1 mice were backcrossed onto inbred FVB mice (F1B1), *neu* transgenic females developed mammary tumors with approximately a 35% incidence at 14 months.

Figure 9. Scatter Plot of Tumor Latencies in *Neu* Proto-Oncogene Transgenic Female Mice.
Tumor latencies for inbred FVB, FVB x C57Bl/6 F1 (F1) and [FVB x C57Bl/6]F1 x FVB (F1B1) virgin female mice.

Figure 10. Expression of the Neu Proto-Oncogene in Mammary Glands of Virgin Female Mice.
To compare the expression levels of the neu proto-oncogene in the normal mammary glands FVB and FVB x C57Bl/6 F1 mice, neu transgene positive virgin female mice from each condition (n=2) were housed together for 1 month to allow for synchronization of estrous cycles. Animals were terminated at 3 months of age and mammary glands were removed, fixed in methacarn and sectioned for immunohistochemistry. Tissue sections were stained with anti-neu antibody. Although expression levels varied in all glands examined, both FVB and FVB x C57Bl/6 F1 mice exhibited strong staining of some ductal elements.

Figure 11. *Neu* Proto-Oncogene-Induced Mammary Tumor Growth Rate in FVB and FVB x C57Bl/6 F1 Mice.
Inbred FVB and FVB x C57Bl/6 F1 female mice transgenic for the *neu* proto-oncogene (n=10 per group) were palpated bi-weekly for the development and growth of mammary tumors. Day 0 was defined as the last palpation before tumor growth was noted. To directly compare tumor growth rate in these two groups, the data was logarithmically transformed and a best fit linear regression was estimated for each tumor. When the slopes of these lines were compared, tumors in F1 mice grew significantly faster than tumors growing in pure strain FVB mice ($p<0.01$)

Figure 12. Expression of the Neu Proto-Oncogene in Mammary Tumors.
To compare the expression levels of the neu proto-oncogene in mammary tumors from FVB and FVB x C57Bl/6 F1 mice, mammary tumor lysates from neu transgene mice were run on SDS-PAGE , transferred to a nitrocellulose membrane and stained with anti-neu antibodies. The

membrane was subsequently washed and reprobed with an anti-actin antibody to control for protein loading. All tumors expressed high levels of the neu protein.

Figure 1.

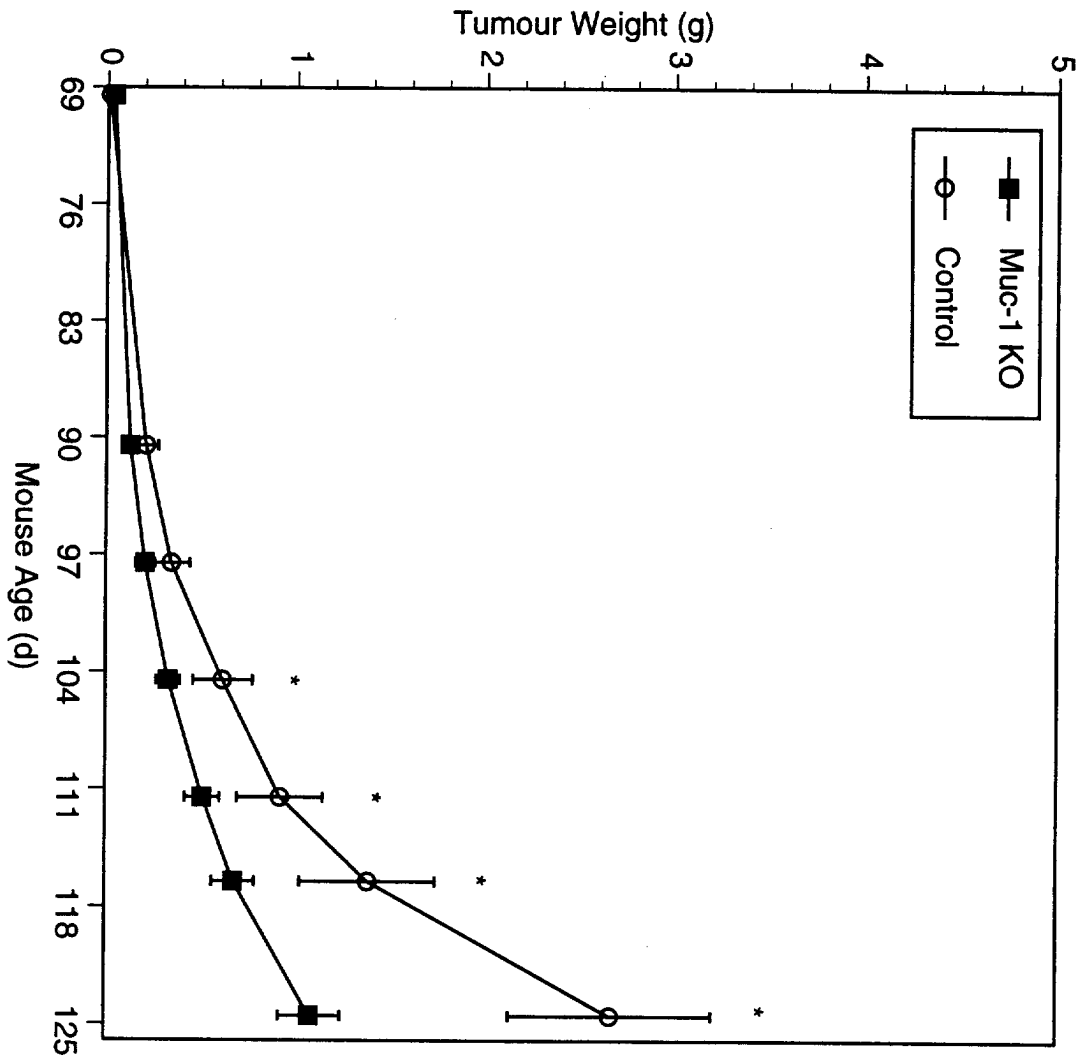

Tumour Weight (g)
0
1
2
3
4
5
Mouse Age (d)
69
76
83
90
97
104
111
118
125
Muc-1 KO
Control
*
*
*
*

Figure 2.

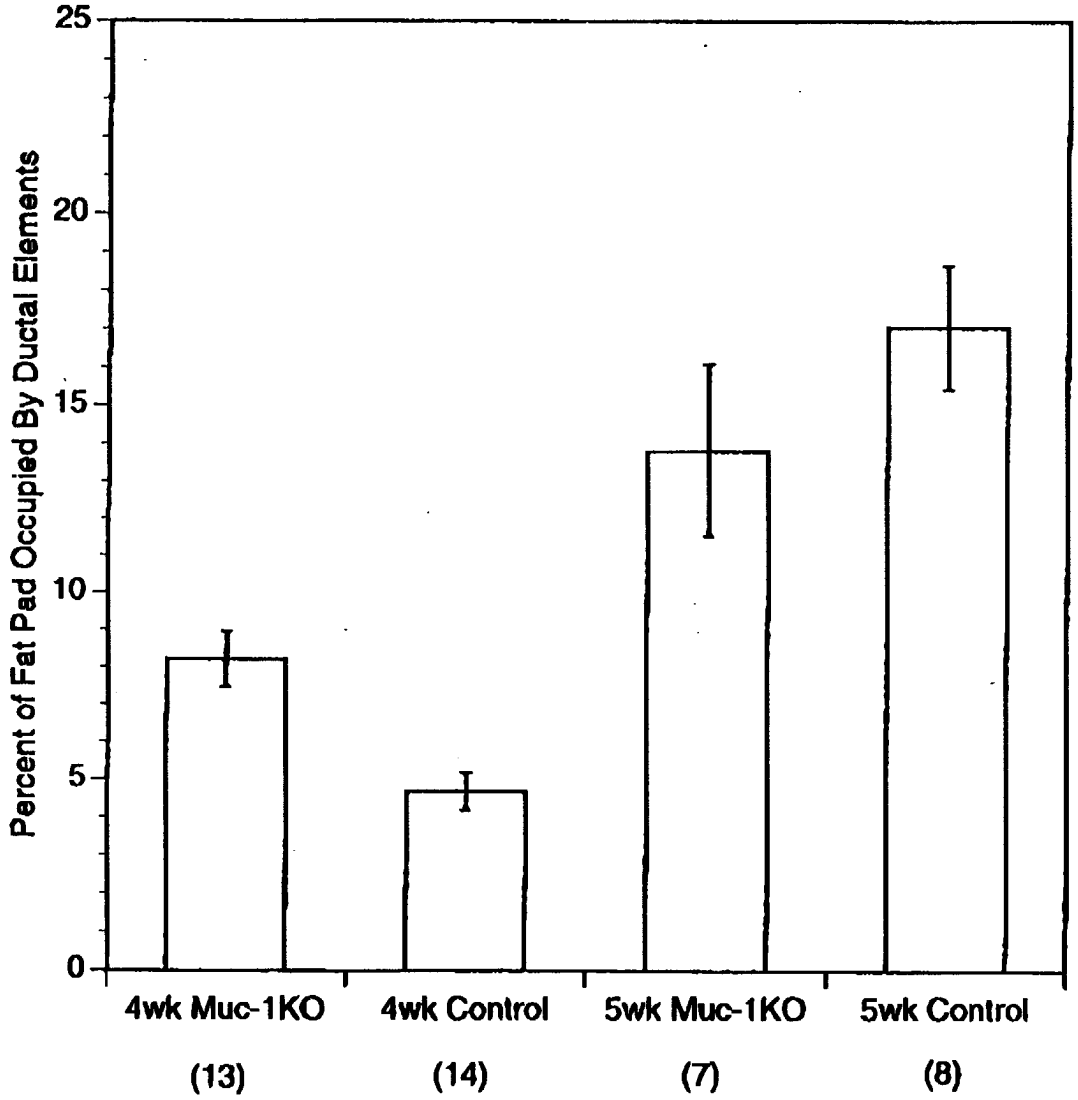

Percent of Fat Pad Occupied By Ductal Elements
25
20
15
10
5
0
4wk Muc-1KO
4wk Control
5wk Muc-1KO
5wk Control
(13)
(14)
(7)
(8)

Figure 3.

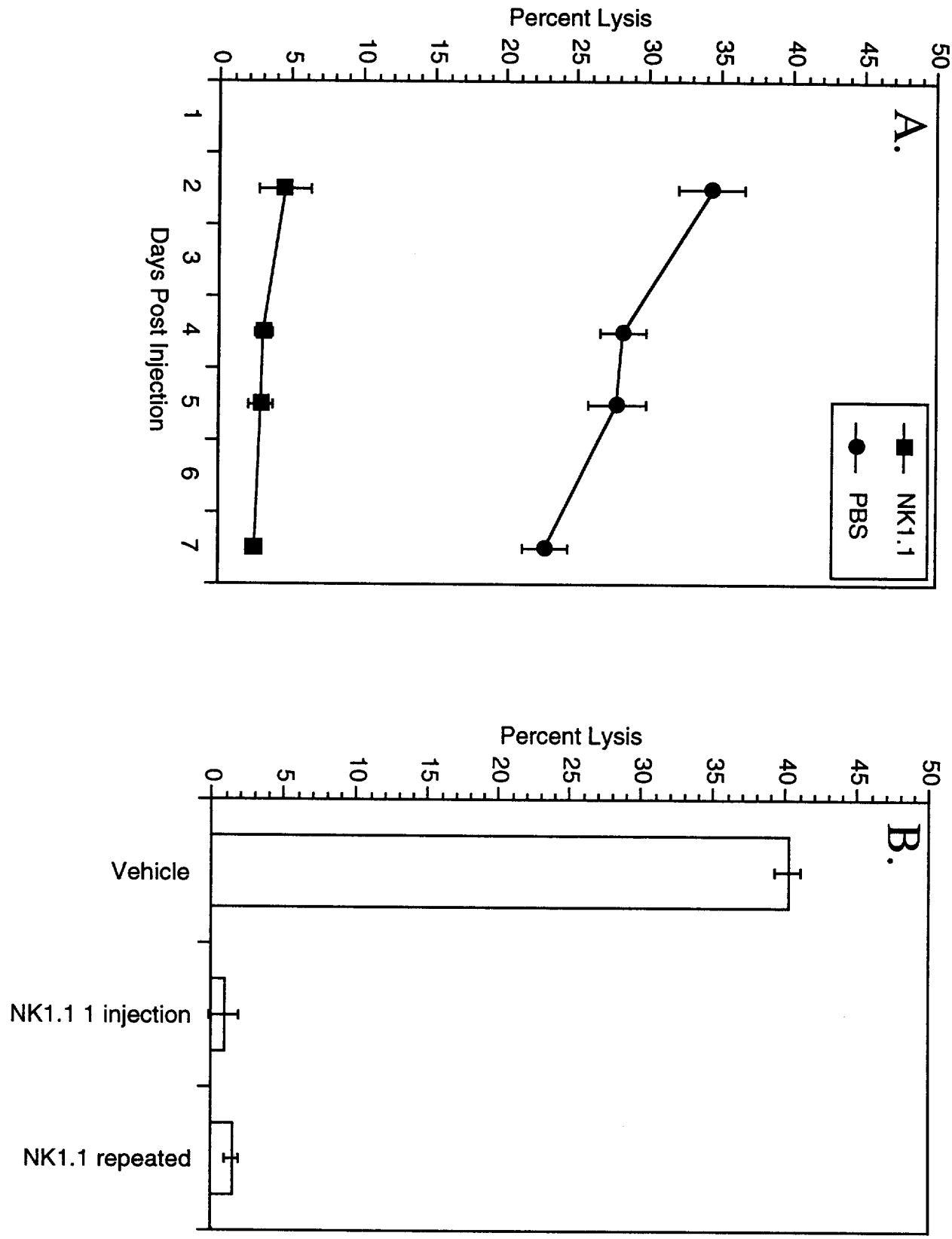

A.
Percent Lysis
0
5
10
15
20
25
30
35
40
45
50
Days Post Injection
1
2
3
4
5
6
7
NK1.1
PBS
B.
Percent Lysis
0
5
10
15
20
25
30
35
40
45
50
Vehicle
NK1.1 1 injection
NK1.1 repeated

Figure 4.

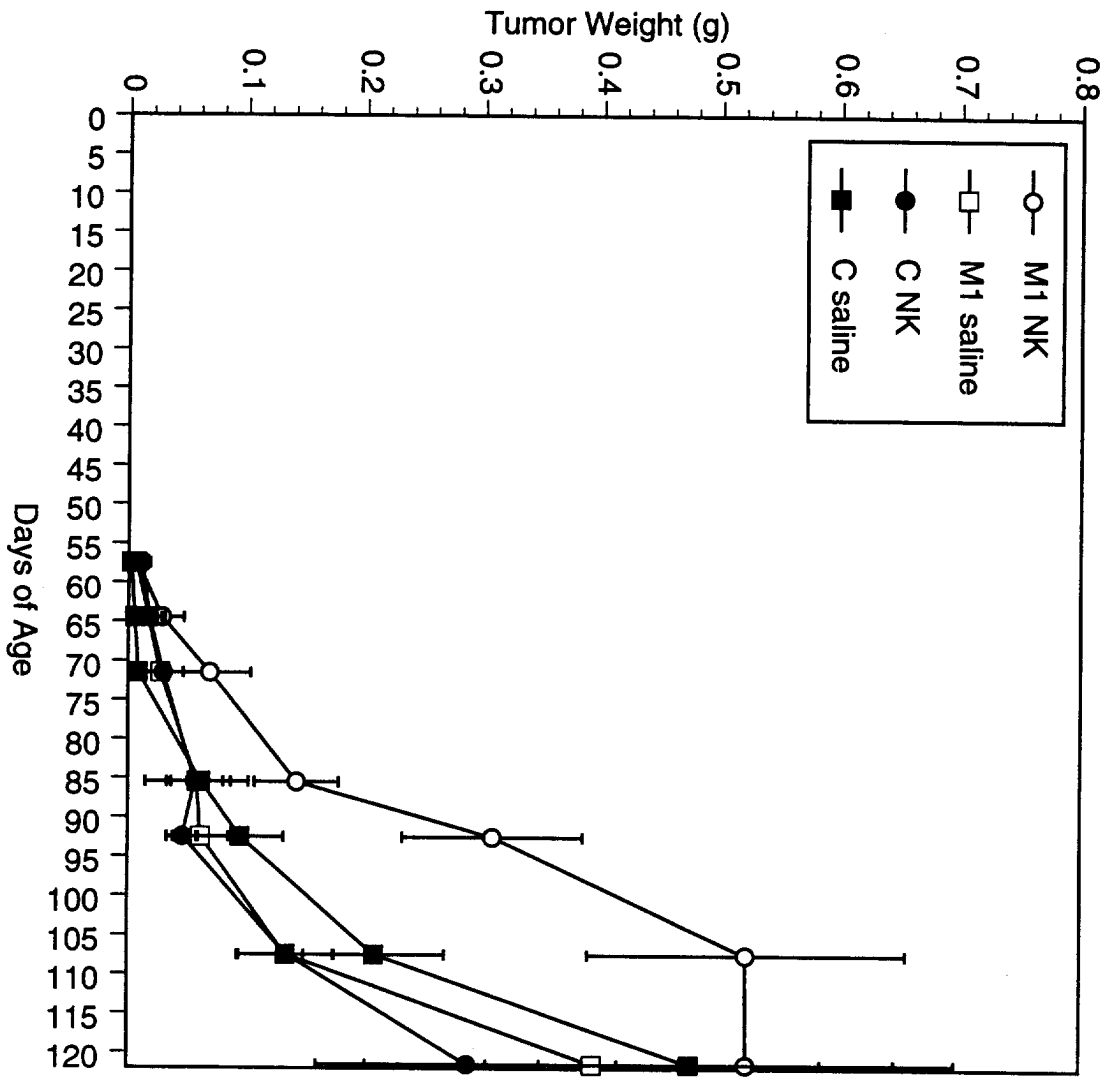

Tumor Weight (g)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Days of Age
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
M1 NK
M1 saline
C NK
C saline

Figure5.

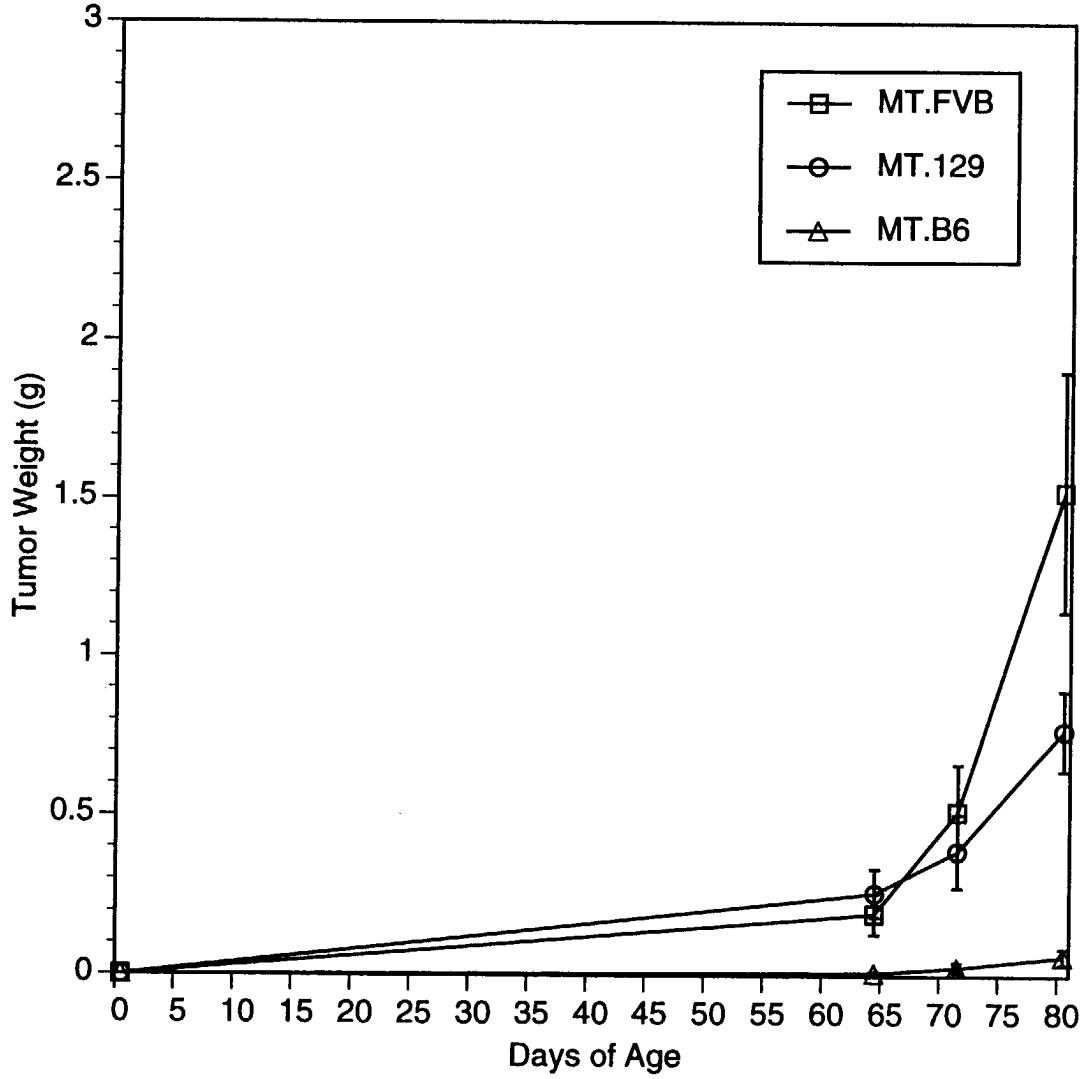

MT.FVB
MT.129
MT.B6
Tumor Weight (g)
Days of Age
0
0.5
1
1.5
2
2.5
3
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80

Figure 6.

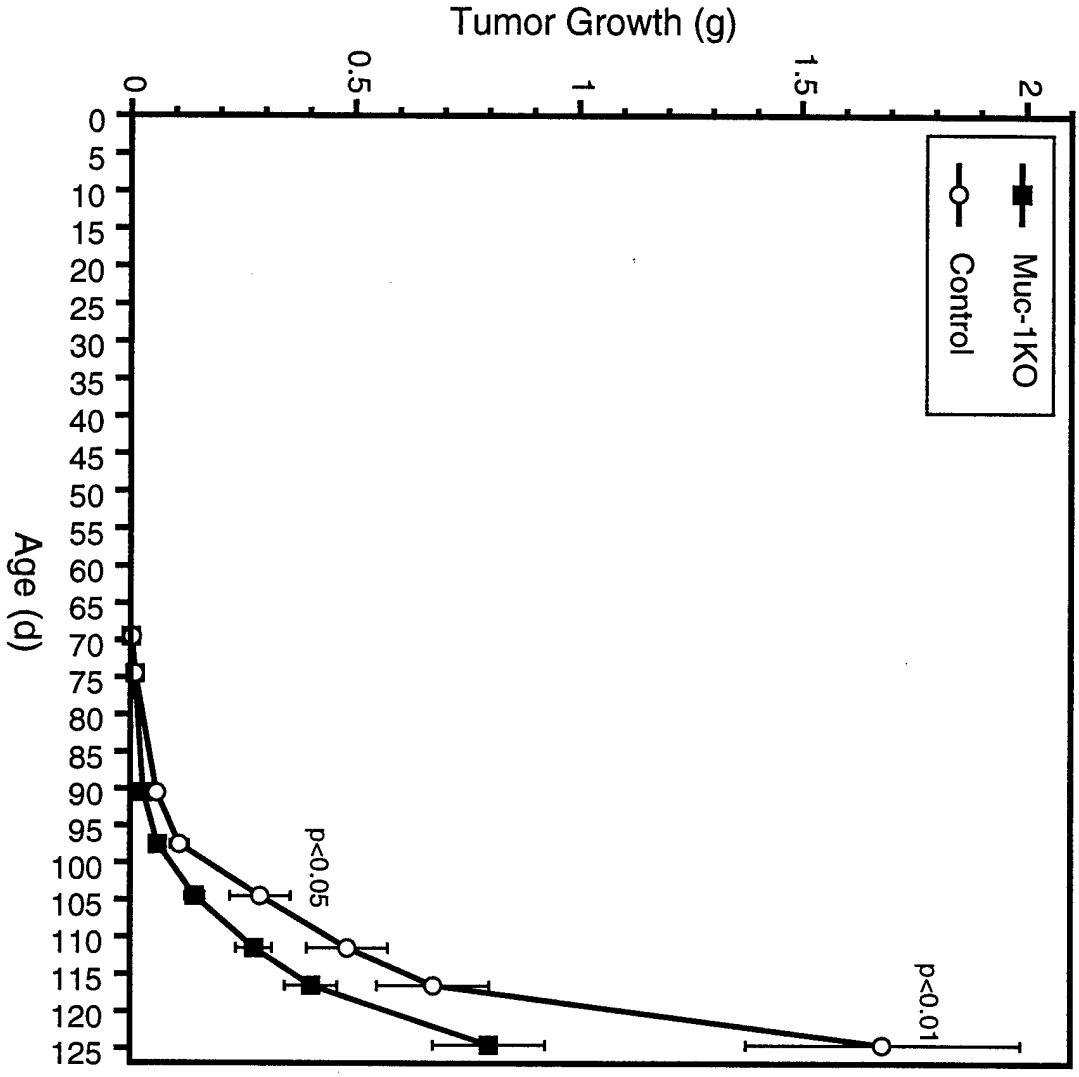

Tumor Growth (g)
0
0.5
1
1.5
2
Age (d)
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
Muc-1KO
Control
p<0.05
p<0.01

Figure7.

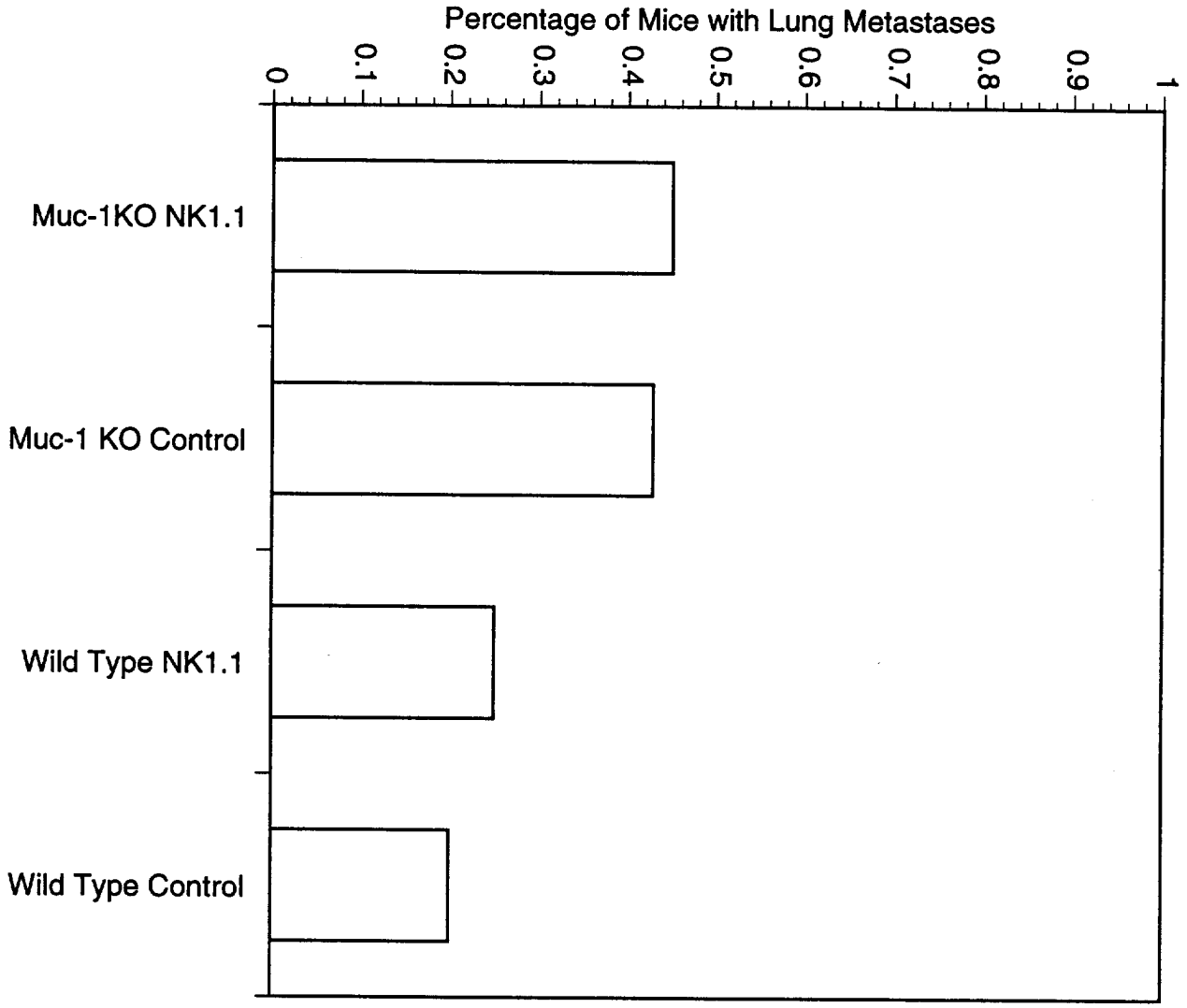

Percentage of Mice with Lung Metastases
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Muc-1KO NK1.1
Muc-1 KO Control
Wild Type NK1.1
Wild Type Control

Figure 8.

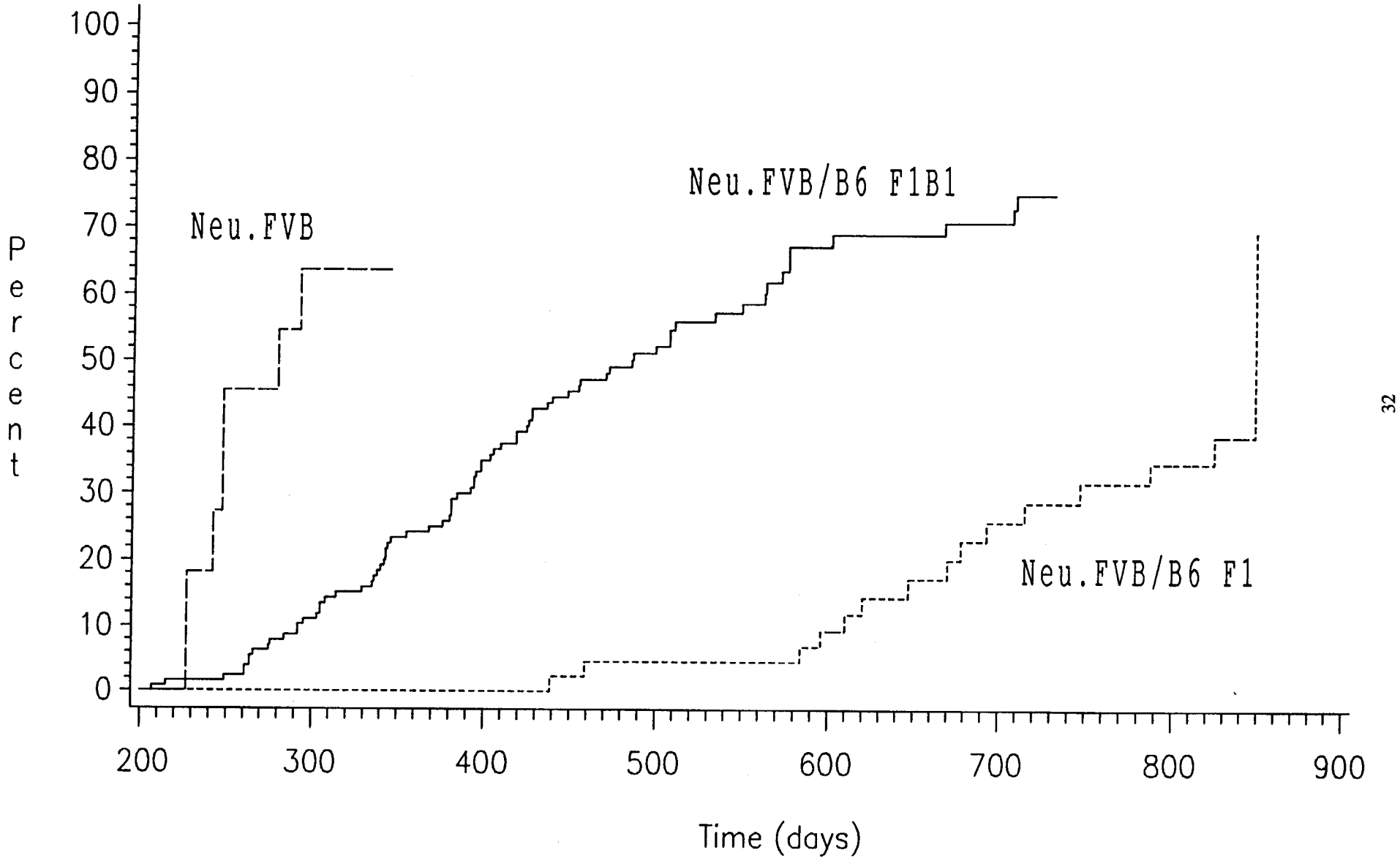

100
90
80
70
60
50
40
30
20
10
0
Percent
Neu.FVB
Neu.FVB/B6 F1B1
Neu.FVB/B6 F1
200
300
400
500
600
700
800
900
Time (days)

Figure 9.

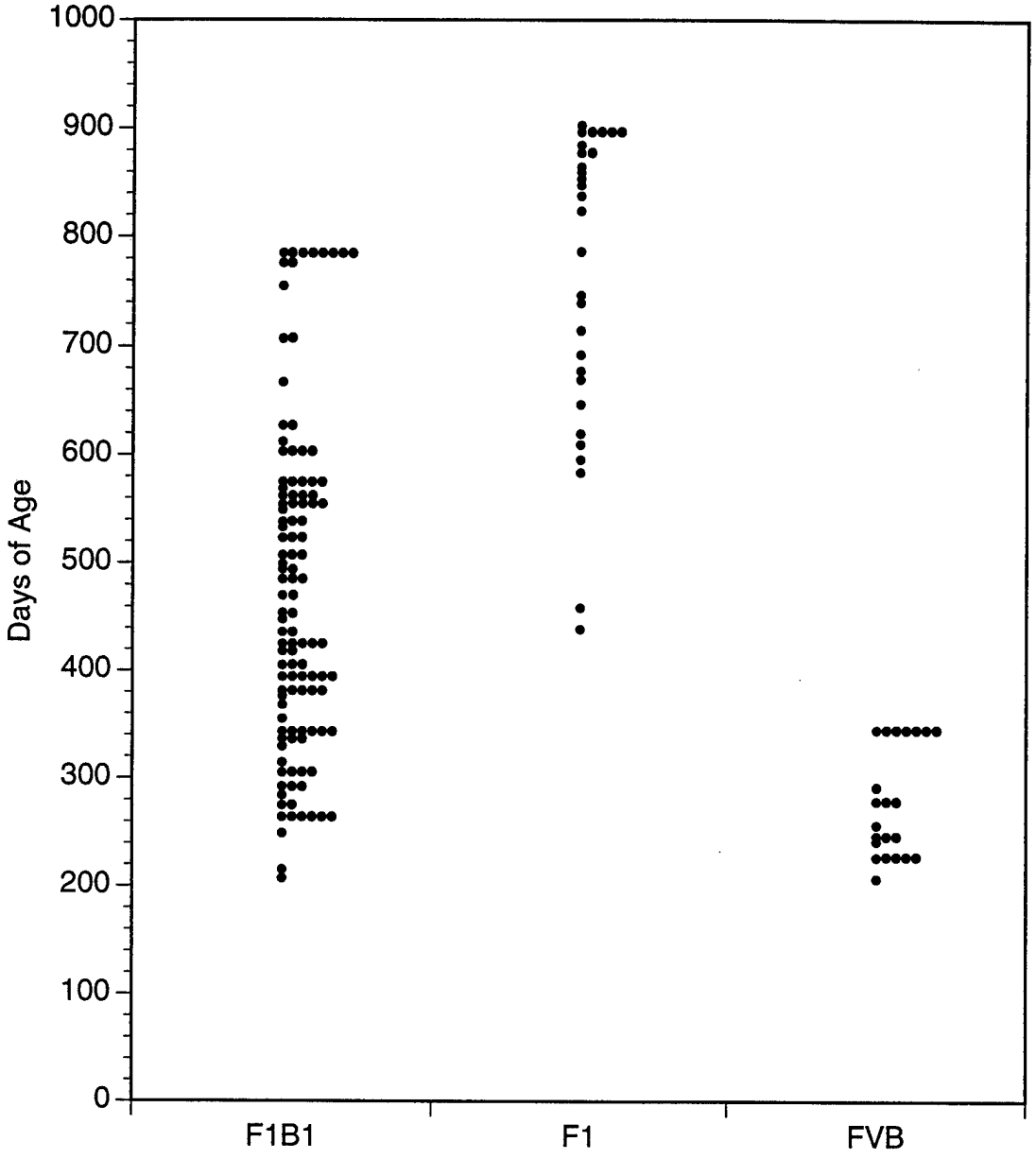

1000
900
800
700
600
500
400
300
200
100
0
Days of Age
F1B1
F1
FVB

Figure 10.

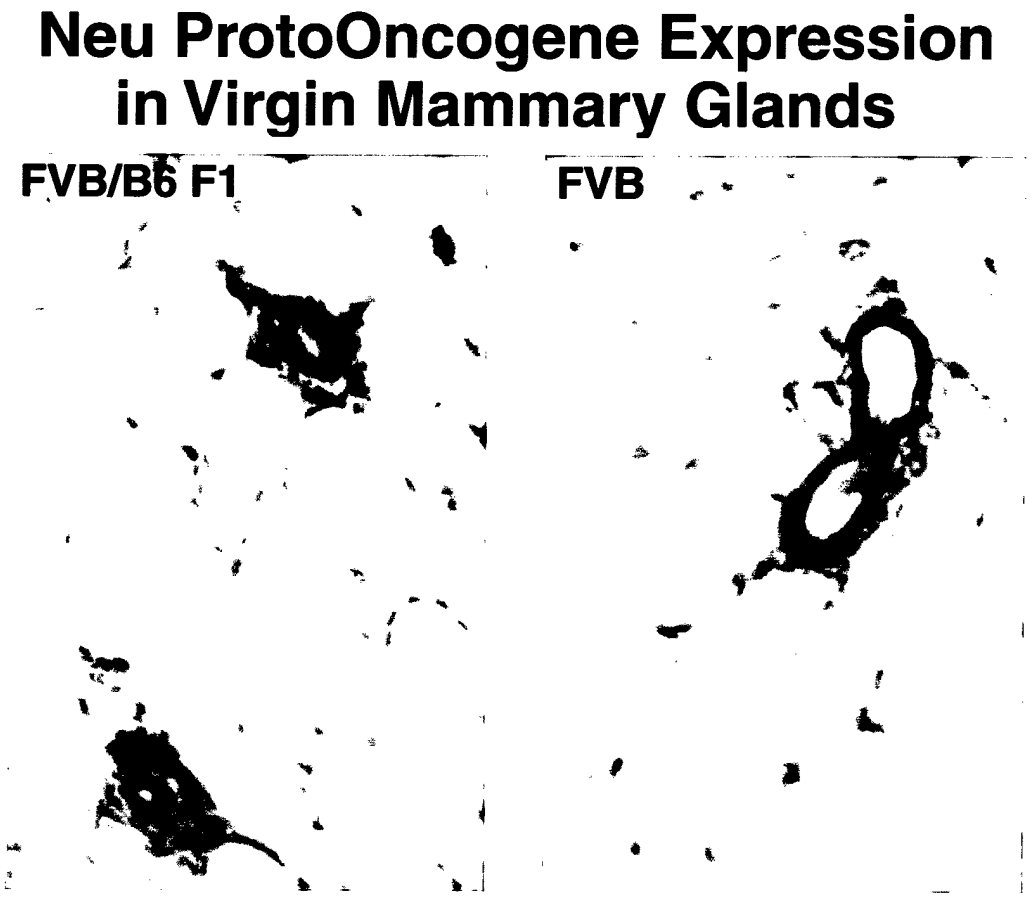

Neu ProtoOncogene Expression
in Virgin Mammary Glands
FVB/B6 F1
FVB

Figure 11.

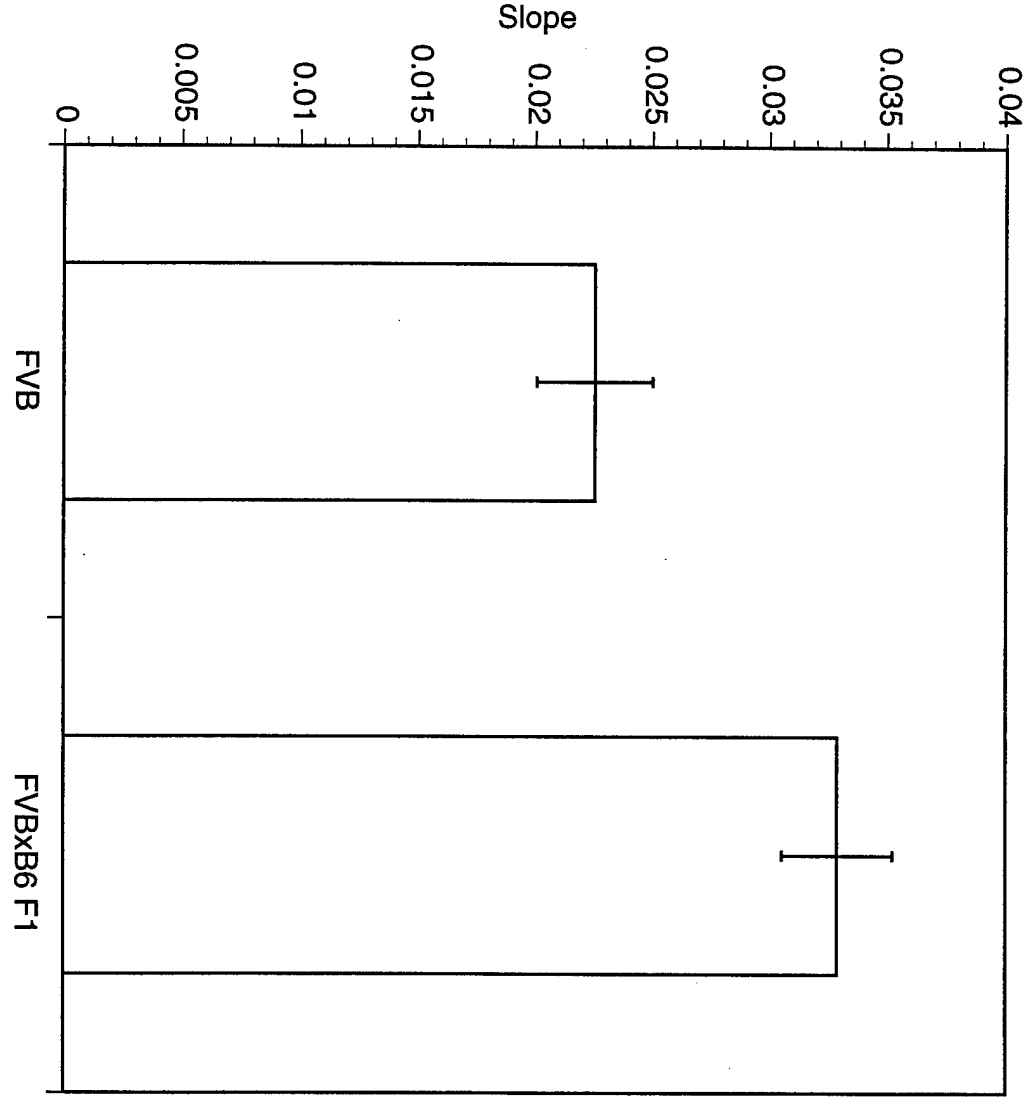
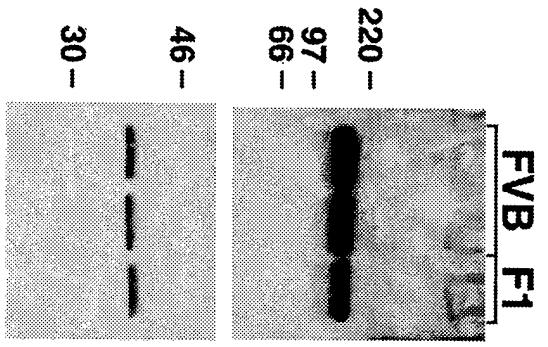


Figure 12.



June 26, 1997
Gerald J. Rowse, Ph.D.

Revised Statement of Work

Progression and Metastasis of Mammary Carcinomas: Potential Role of the Muc-1 Glycoprotein

Status

To make mutant mice of homozygous strains lacking the Muc-1 gene:

a. Chimeric mice mutant for the Muc-1 gene will be crossed with 129sv and the heterozygous progeny intercrossed to produce homozygous mutant mice on a pure 129sv background (months 1-3). ..... Complete

b. Heterozygotes will be backcrossed onto C57Bl/6 line through 12 generations to produce homozygous mutant mice on a pure C57Bl/6 background (months 1-36). ..... Complete

Specific Aim 1, Analysis of the effects of Muc-1 gene mutations on epithelial organogenesis (months 3 to 36).

a. Histological analysis of embryo and adult mice (months 3-12). ..... Complete

b. Whole mount analysis of mammary gland development in C57Bl/6 inbred mice (months 24-36). ..... In Progress

Specific Aim 2, Analysis of the effects of Muc-1 gene deletion on tumor formation and progression (months 6 to 48).

a. Polyoma virus middle T antigen (Mtag) induced tumors in outbred transgenic mice (months 6-18). ..... Complete

b. Effects of Muc-1 gene deletion on MTag induced mammary tumor cell proliferation and apoptosis. (months 24-48). ..... In Progress

c. Effects of Muc-1 gene deletion on anti-tumor activity of natural killer cells in an MTag induced mammary tumors in MTag transgenic mice. (months 24-48). ..... In Progress

Specific Aim 3, Analysis of the effect of Muc-1 gene deletion on metastasis of mammary gland tumors (months 18-48).

a. Effects of Muc-1 gene deletion on MTag induced mammary tumor metastasis to the lung in MTag transgenic mice. (months 18-24). ..... Complete

b. Effects of Muc-1 gene deletion on anti-metastatic activity of natural killer cells in an MTag induced mammary tumor model utilizing MTag transgenic mice. (months 24-48). ..... In Progress

Specific Aim 4, Characterization of tumor suppressive loci acting on mammary tumorigenesis induced by the overexpression of the neu proto-oncogene in neu transgenic mice. (months 18-48). ..... In Progress

Gerry J. Rowse
Mayo Clinic Scottsdale, Scottsdale, AZ

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Rowse, GJ, Ritland, SR, Gendler, SJ. The Influence of Genetic Background om Mouse Mammary Tumorigenesis in MMTV-neu Transgenic Mice. 1997; Manuscript in preparation.

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